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Arbuscular mycorrhizal fungi colonize nonfixing root nodules of several legume species

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Summary

• Many legumes form tripartite symbiotic associations with rhizobia and arbuscular mycorrhizal fungi (AMF). Rhizobia are located in root nodules and provide the plant with fixed atmospheric nitrogen, while AMF colonize plant roots and deliver several essential nutrients to the plant. Recent studies showed that AMF are also associated with root nodules. This might point to interactions between AMF and rhizobia inside root nodules.

• Here, we test whether AMF colonize root nodules in various plant–AMF combinations. We also test whether nodules that are colonized by AMF fix nitrogen.

• Using microscopy, we observed that AMF colonized the root nodules of three different legume species. The AMF colonization of the nodules ranged from 5% to 74% and depended on plant species, AMF identity and nutrient availability. However, AMF-colonized nodules were not active, that is, they did not fix nitrogen.

• The results suggest that AMF colonize old senescent nodules after nitrogen fixation has stopped, although it is also possible that AMF colonization of nodules inhibits nitrogen fixation.

Key words: mutualism, mycorrhiza, nitrogen fixation, nodule, rhizobium, symbiosis.

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Introduction

Arbuscular mycorrhizal fungi (AMF) and rhizobia are two of the most important plant symbionts. They play a key role in natural ecosystems and influence plant productivity, plant nutrition and plant community structure (Grime *et al.*, 1987; van der Heijden *et al.*, 1998; Cleveland *et al.*, 1999; van der Heijden *et al.*, 2006). The majority of legumes form symbiotic associations with both phosphorus-acquiring AMF and nitrogen (N)-fixing rhizobia (Werner, 1992; Smith & Read, 1997; Sprent, 2001; Lodwig *et al.*, 2003). The AMF colonize the root cortex and rhizobia are located in root nodules, which are specialized root structures that are formed on plant roots after a complex molecular dialogue between rhizobia and the host plant (Schultze & Kondorosi, 1998; Ferguson & Mathesius, 2003).

The dual symbiosis with AMF and rhizobia is crucial for legume growth within plant communities (van der Heijden *et al.*, 1998, 2006). In pot experiments as well as field experiments co-inoculation with both symbionts resulted in higher plant biomass and better N and phosphorus (P) acquisition, although these effects were also dependent on the specific symbiont combination (Azcón-Aguilar & Barea, 1981; Rao *et al.*, 1986; Azcón *et al.*, 1991; Requena *et al.*, 2001; Xavier & Germida, 2002). Arbuscular mycorrhizal fungi can support nitrogen fixation by providing legumes with P and other immobile nutrients that are essential for N fixation, such as copper (Cu) and zinc (Zn) (Li *et al.*, 1991; Kothari *et al.*, 1991; Clark & Zeto, 2000). As a consequence N fixation can be reduced or even completely inhibited in the absence of AMF at low nutrient availability (Asimi *et al.*, 1980; Azcón *et al.*, 1991).

The N fixation process takes place inside the root nodules. Early reports stated that AMF are not present in root nodules (Crush, 1974; Harley & Smith, 1983), but others have found AMF colonization of root nodules (Baird & Caruso, 1994; Vidal-Dominguez *et al.*, 1994). Moreover, molecular identification revealed that under natural circumstances, root nodules are colonized by specific AMF communities that are similar

in different legume species and different from the root AMF community (Scheublin *et al.*, 2004). Whether AMF communities represent abundant colonization of the nodules or hyphae on the surface of the nodules cannot be derived from molecular data alone. The presence of AMF in the nodules could indicate that AMF deliver nutrients that are essential for N fixation directly into the nodules. However, it is not yet clear whether the presence of AMF in the nodules influences nodule functioning, and whether AMF-colonized nodules are active (i.e. fixing N).

It has been shown that AMF colonization in roots can be influenced by plant species, AMF identity and nutrient availability (Smith & Read, 1997). High P levels for example have been shown to decrease AMF colonization in roots (Koide, 1985). However, it is not known whether AMF colonization in root nodules is also affected by these factors. In a previous study (Scheublin *et al.*, 2004), nodules of three different legume species from the field appeared to be colonized by a limited number of AMF types. Therefore a preference for certain AMF types might exist, and root nodule colonization could be dependent on AMF type.

In this study we investigated whether the presence of AMF in root nodules is related to nodule functioning. We tested whether AMF are able to colonize root nodules in different plant–AMF combinations, whether AMF-colonized nodules fix N, and whether nodule colonization is influenced by plant species, AMF identity or nutrient availability.

Materials and Methods

Biological material

Dune sand, rhizobia and four of the five AMF isolates used in this study originated from Dutch dry dune grassland (Provinciale Waterleidingduinen; 52°36′N, 4°38′W). Rhizobia were isolated from nodules of *Trifolium repens* L. (strain T.r. 2), *Ononis repens* L. (strain O.r. 2) and *Lotus corniculatus* L. (strain L.c. 2) that were collected at the field site. The characteristics of these strains are described elsewhere (van der Heijden *et al.*, 2006). The AMF isolates Dutch Dunes 1 (DD-1), DD-2 and DD-3 originated from single spores and DD-5 originated from a sporocarp. These spores were obtained from trap cultures with *T. repens* inoculated with nodulated legume root fragments from the field (DD-1, DD-2) or *Hieracium pilosella* plants from the field grown in sterile dune sand (DD-3, DD-5). The fifth AMF isolate, BEG 21, originated from Swiss calcareous grassland (van der Heijden *et al.*, 1998). A portion of the small-subunit ribosomal DNA of all AMF isolates was amplified with the PCR primers NS31 and AM1 (Simon *et al.*, 1992; Helgason *et al.*, 1998) and subsequently sequenced. The AMF isolates DD-2, DD-3, DD-5 and BEG 21 belong to the Glo8 AMF type, which constitutes 63% of the AMF communities in root nodules in the field (Scheublin *et al.*, 2004). DD-1 belongs to a group with Glo18, Glo48 and Glo53, which together constitute 0.6% of the AMF communities in field nodules. Sequences have been deposited in the GenBank database under accession numbers DQ377988 to DQ377991 and DQ487217. We used sand–root mixtures of dried pot cultures of each isolate as inoculum for the experiments. Seeds of *T. repens*, *O. repens* and *L. corniculatus* were obtained from a native seed supplier (Cruydt-hoeck, Groningen, the Netherlands). Seeds were surface-sterilized in 2% chloride for 10 min, thoroughly rinsed with sterile water and germinated on 1.6% water–agar. *Ononis repens* was incubated in sulphuric acid for 8 min before sterilization in order to induce germination. Plants were grown in a climate-controlled glasshouse, with a day temperature of 22°C, a night temperature of 15°C, and a day : night cycle of 14 h : 10 h. In addition to natural light, during the day-period light was supplied by 400 W HPI-T lamps (Philips, Eindhoven, the Netherlands) at 1.2 m above plant height.

Experiment 1: effects of plant species and AMF identity on nodule colonization of *T. repens* and *O. repens*

Trifolium repens and *O. repens* plants were either inoculated with AMF isolate DD-5 or BEG 21, or left uninoculated. Four replicates per treatment resulted in a total of 24 pots. Eight-hundred millilitre pots were filled with 800 g of air-dried dune sand, mixed with 10% demineralized water and autoclaved for 2 h at 110°C. Twenty-five grams of AMF inoculum was mixed through each pot. The nonAMF treatment received autoclaved inoculum. A microbial wash was prepared by wet-sieving 333 g of each inoculum through a series of sieves into a final volume of 1 l. The finest sieve was 10 μm. Each pot received 30 ml of the microbial wash. Rhizobial suspensions with an optical density at 580 nm (OD_{580}) of 0.2 were prepared from strains T.r. 2 and O.r. 2, and all pots received 1 ml of each suspension in order to ensure nodulation. Six germinated seeds were planted into each pot, and after 10 d the surviving seedlings were reduced to three per pot. The experiment lasted for 1 yr, from May 2004 till May 2005. Between week 6 and week 15 each pot received 5 ml of a modified Hoagland nutrient solution (6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 0.75 mM NH_4NO_3, 0.5 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA) weekly to a total of 50 ml (Hoagland & Arnon, 1950). The plants were watered regularly and the water content was kept between 10% and 20% of the soil dry weight.

Experiment 2: effects of nutrient availability and AMF identity on nodule colonization of *L. corniculatus*

Lotus corniculatus plants were inoculated with one of four AMF isolates, DD-1, DD-2, DD-3 or BEG 21, and received

either a low-N or a low-P nutrient solution. Five replicates per treatment resulted in a total of 40 pots. Dune sand was mixed with 10% demineralized water. Sand was autoclaved for 2 h at 110°C. After autoclaving, available (KCl-extractable) N was 2.1 mg kg^{-1} and available ($NaHCO_3$-extractable) phosphorus 0.41 mg kg^{-1}. Eight-hundred millilitre pots were filled with 750 g of sand (dry weight) and 25 g of AMF inoculum. A microbial wash was prepared by wet-sieving 750 g of each inoculum and 750 g of field soil through a series of sieves into a final volume of 2.5 l. The finest sieve was 10 μm. Each pot received 10 ml of the microbial wash and 1 ml of a rhizobial suspension of strain L.c. 2 with an OD_{580} of 0.2. Four germinated seeds were planted into each pot, and during the first 3 wk, dead seedlings were replaced by seedlings of the same age that were grown in sterile sand.

Plants were supplied with one of two nutrient solutions that were based on the Hoagland solution (Hoagland & Arnon, 1950). One nutrient solution, the low-N solution, had an N : P ratio of 3.6 (6 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 3.5 mM $CaCl_2$, 1 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA). The other nutrient solution, the low-P solution, had an N : P ratio of 18 (6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 0.8 mM NH_4NO_3, 0.4 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $(NH4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA). The experiment lasted for 10 months, from August 2004 until June 2005. After 5 wk each pot received 5 ml of nutrient solution weekly to a total of 200 ml at the end of the experiment. The water content was kept between 10% and 20% of the soil dry weight.

Harvest

Roots were washed and kept between moist tissue paper. Nodules were processed within 1 h after harvest. Nitrogen fixation was determined for 15 nodules per pot for three replicates of each treatment. These 15 nodules were chosen to include morphologically different nodules: white, pink and brown nodules, hard and soft, and of various sizes. NonAMF treatments were not tested for N-fixing activity. A total of 90 nodules were tested for *T. repens*, 90 for *O. repens* and 357 for *L. corniculatus*. Each nodule, attached to about 0.5 cm of root, was placed into a 2 ml vial directly after cutting it from the root system. Acetylene was added to the vial to a concentration of 10%, and after 1 h 0.25 ml of gas was analysed for ethylene on a gas chromatograph (Hewlett Packard 5890 A; Avondale PA, USA) fitted with a Poropak N column (10 ft, 1/8 inch, 80/100 mesh; Supelco, Bellefonte PA, USA), using an oven temperature of 90°C and the flame ionization detector (FID) at 275°C. In each series of 15 nodules, three control vials without nodules were analysed. The amount of ethylene formed was determined, which gives an indication for the amount of N fixation (Hardy *et al.*, 1968). Subsequently, each nodule was weighed and stored at 4°C before staining. Nitrogen-fixing activity was expressed as the amount of ethylene formed per minute per gram nodule (fresh weight).

In order to determine the percentage of AMF colonized root nodules an additional 20–100 nodules per pot were collected for all replicates and stored at 4°C before staining. All nodules were collected on a subsample of the roots, and therefore these nodules were a representative sample. A total of 387 nodules were collected for *T. repens*, 329 for *O. repens* and 3086 for *L. corniculatus*. Roots were also collected and stored at 4°C before staining.

Roots were cleared in 10% KOH and stained with Trypan blue (Phillips & Hayman, 1970). The modified line intersection method (McGonigle *et al.*, 1990) was used to determine the percentage of root length colonized by AMF. For each sample, 100 intersections were examined. Trypan blue heavily stained the interior of the root nodules, and therefore it was difficult to visualize AMF in root nodules with this stain. Acid fuchsin appeared to be a more suitable stain for nodules. Vesicles and spores were clearly visible after acid fuchsin staining, but hyphae and arbuscules were more difficult to distinguish. Nodules were cleared in 10% KOH for 3 h at 90°C and 10% HCl for 1 h at room temperature, and then stained with acid fuchsin for 1 h at 90°C. The AMF colonized nodules were divided into three categories: few spores per nodule (+; cf. Fig. 1b), completely occupied with spores (+ + +; cf. Fig. 1d,e), and intermediate (+ +; cf. Fig. 1c).

For experiment 2, shoots were dried at 70°C for 3 d. Dried shoots were ground at 30 Hz in a mixer mill (MM200; Retsch, Haan, Germany) for 2 min. Nitrogen concentrations were determined using an elemental analyser (NC2500; ThermoQuest Italia, Rodana, Italy), coupled with a continuous-flow isotope ratio mass spectrometer (Delta Plus; ThermoQuest Finnigan, Bremen, Germany). Phosphorus concentrations were determined by digestion of ground plant material with a HCl–HNO_3 (1 : 4) solution at 140°C for 7 h in Teflon pressure bombs, followed by colorimetric determination of phosphorus with the molybdate blue ascorbic acid method (Murphy & Riley, 1962).

Statistical analysis

For the statistical analyses we used SPSS, version 10.1, and the significance level was 0.05. In experiment 1, differences in nodule colonization were tested in a two-way ANOVA with plant species and AMF type as the two factors. Colonization levels in the nonAMF treatment were zero, and excluded from the ANOVA. In experiment 2, differences in nodule colonization were tested in a two-way ANOVA with AMF type and nutrient regime as the two factors. In experiment 2 an 'arcsin' transformation was necessary to obtain homogeneous variances, which is one of the requirements for an ANOVA analysis. Linear regression analysis was performed to investigate

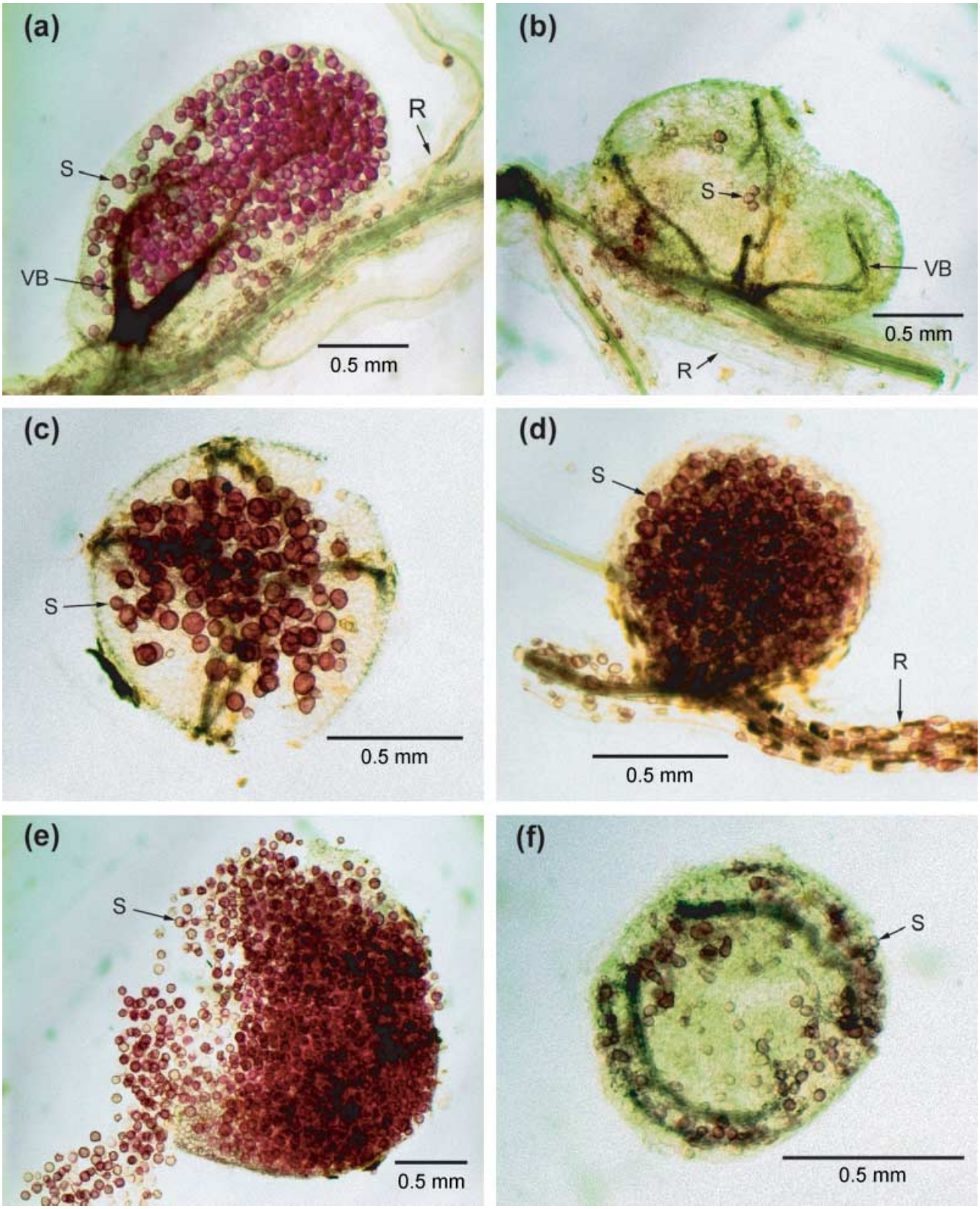

Fig. 1 Arbuscular mycorrhizal fungal (AMF) colonization of (a) *Trifolium repens* or (b–f) *Lotus corniculatus* root nodules, inoculated with AMF isolate BEG 21 (a–e) or Dutch Dunes 1 (DD-1) (f). S, spore; VB, vascular bundle; R, root.

Table 1 Numbers of nitrogen fixing and/or arbuscular mycorrhizal fungal colonized nodules of three legume species

	Not N-fixing		N-fixing		
	Not colonized	Colonized	Not colonized	Colonized	Total
Trifolium repens (experiment 1)	18	46	26	0	90
Ononis repens (experiment 1)	35	8	47	0	90
Lotus corniculatus (experiment 2)	141	61	155	0	357

Nitrogen-fixation was determined by the acetylene reduction method.

whether a correlation existed between AMF colonization of roots and AMF colonization of nodules.

Results

A total of 537 nodules of three legume species were tested for N-fixing activity and AMF colonization (Table 1). A total of 228 nodules were fixing N and the remaining 309 nodules showed no N-fixing activity. However, none of the N-fixing nodules was infected by AMF. The N-fixation rates (expressed as the amount of ethylene formed per minute per gram nodule) of the fixing nodules ranged from 0.5 to 547 nmol min^{-1} g^{-1}, with an average of 190 nmol min^{-1} g^{-1} for *T. repens*, 117 nmol min^{-1} g^{-1} for *O. repens*, and 39 nmol min^{-1} g^{-1} for *L. corniculatus*. Spores were observed to be the main fungal structure in root nodules, and AMF isolate DD-1 had smaller spores than the other AMF isolates (Fig. 1).

All plant roots in the treatments with AMF were colonized by AMF and contained arbuscules and/or vesicles (Figs 2a and 3a). Root colonization levels were *c.* 90% for *T. repens* and *O. repens* in Expt 1 and ranged between 58% and 100% for *L. corniculatus* in Expt 2. The nonAMF treatments remained free of AMF colonization, both in the roots and in the nodules. The percentage of nodules colonized by AMF varied between 5% and 74% and was dependent on plant species and AMF isolate in experiment 1 (Fig. 2b). Nodule colonization was higher in *T. repens* than in *O. repens* and higher with AMF isolate BEG 21 than with isolate DD-5. In experiment 2, nodule colonization was higher with the low-P nutrient solution than with the low-N solution (Fig. 3b). Nodule colonization was divided into three categories. The most heavily infected category, nodules completely occupied with spores, was almost exclusively found with AMF isolate BEG 21 in both experiments.

Plants that received the low-N nutrient solution in experiment 2 had significantly lower N concentrations (1.3% vs 1.6% on average; $F_{1,32} = 63.18$; $P < 0.001$, two-way ANOVA) and significantly higher phosphorus concentrations (0.039% vs 0.029% on average; $F_{1,32} = 34.08$; $P < 0.001$, two-way ANOVA) than plants that received the low-P nutrient solution. AMF colonization of both the roots and the nodules was significantly higher with the low-P nutrient solution. There was no correlation between root and nodule colonization ($R^2 = 0.022$; $F_{1,38} = 0.86$; $P = 0.361$).

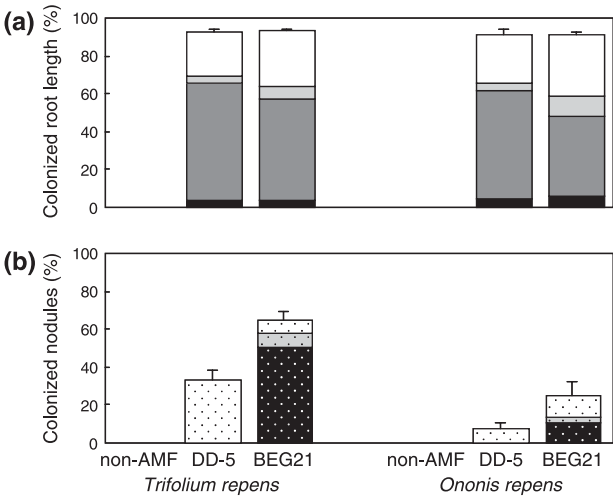

Fig. 2 Percentage of (a) root length and (b) of nodules colonized by arbuscular mycorrhizal fungi (AMF) in experiment 1. The percentage of AMF colonized nodules is dependent on plant species ($F_{1,12} = 34.49$; $P < 0.001$) and AMF isolate ($F_{1,12} = 20.01$; $P = 0.001$), and there is no interaction between these two variables ($F_{1,12} = 2.72$; $P = 0.125$) in a two-way ANOVA. AMF structures in the roots are divided into four categories: hyphae (open column); arbuscules (light tint); vesicles (dark tint); arbuscules + vesicles (closed column). The AMF abundance in nodules is divided into three categories: few spores per nodule (open stippled column; cf. Fig. 1b); completely occupied with spores (closed stippled column; cf. Fig. 1d,e); and intermediate (tinted stippled column; cf. Fig. 1c).

Discussion

This study confirms that AMF are capable of nodule colonization in legumes (Baird & Caruso, 1994; Vidal-Dominguez *et al.*, 1994). Up to 74% of the nodules in our experiments contained AMF. However, AMF-colonized nodules never fixed N, which shows that these nodules were not functional. Moreover, spores, which are the reproduction structures of AMF, were abundantly present in nodules. Hence, colonization of the nodules by AMF is apparently not directly

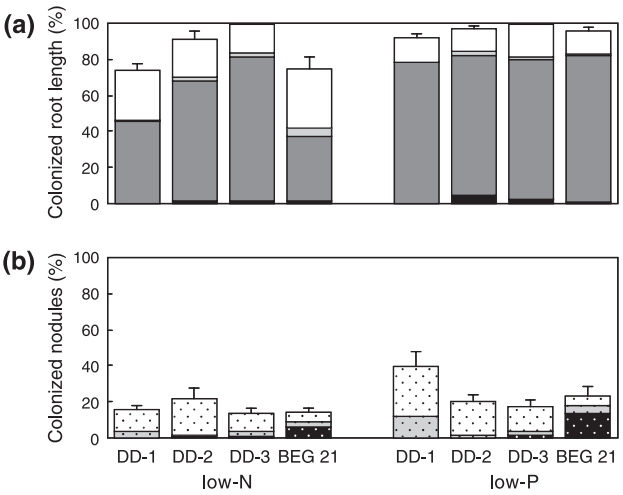

Fig. 3 Percentage of (a) root length and (b) of nodules colonized by arbuscular mycorrhizal fungi (AMF) in experiment 2. *Lotus corniculatus* plants received either a low nitrogen (low-N) or a low phosphorus (low-P) nutrient solution. The percentage of AMF colonized nodules is dependent on nutrient solution ($F_{1,32} = 5.63$; $P = 0.024$), not dependent on AMF isolate ($F_{3,32} = 2.33$; $P = 0.093$), and there is no interaction between these two variables ($F_{3,32} = 2.09$; $P = 0.121$) in a two-way ANOVA. AMF structures in the roots are divided into four categories: hyphae (open column); arbuscules (light tint); vesicles (dark tint); arbuscules + vesicles (closed column). The AMF abundance in nodules is divided into three categories: few spores per nodule (open stippled column; cf. Fig. 1b); completely occupied with spores (closed stippled column; cf. Fig. 1d,e); and intermediate (tinted stippled column; cf. Fig. 1c).

involved in support of N fixation. Stimulation of N fixation by AMF, which has been reported by several studies (Asimi *et al.*, 1980; Azcón *et al.*, 1991), is probably an indirect effect and a result of increased nutrient supply to the whole plant.

Our data suggest either that AMF colonize old senescent nodules after N fixation has stopped, or that AMF colonization of nodules inhibits N fixation. The latter explanation is unlikely, but cannot be excluded based on these data. From an AMF perspective, colonization of root nodules could be favourable when nodules provide a protective environment for AMF sporulation (Vidal-Dominguez *et al.*, 1994). Alternatively, AMF might be attracted by the high N concentrations of the nodule tissue. Arbuscular mycorrhizal fungi contained up to 3.7 times greater N concentrations than plants, indicating that AMF have a high N requirement (data not shown). However, it is not clear whether AMF acquired N from the soil and/or from (dead or alive) plant tissue.

From the plant's perspective, AMF colonization of senescent nodules could also be favourable because AMF might protect senescent nodules from pathogen attack and because nodules could act as mycorrhizal inoculum for future plant generations. Conversely, AMF colonization of nodules might negatively affect the fitness and inoculum potential of rhizobia from the nodules. Interestingly, inoculation of plants with root nodules from the field resulted in abundant AMF colonization (data not shown). This indicates that nodules can act as inoculum source both for AMF and rhizobia.

Establishment of AMF and rhizobia symbioses have many similarities and share important steps during symbiont recognition (Albrecht *et al.*, 1999; Staehelin *et al.*, 2001; Provorov *et al.*, 2002). Rhizobial signals (Nod factors) have been shown to stimulate AMF colonization (Xie *et al.*, 1995). Whether these signals play a role in AMF colonization of nodules is unclear, but this is unlikely if AMF colonize senescent nodules.

Nodule colonization was dependent on plant species, AMF identity and nutrient availability. Nodule colonization was higher under low phosphorus circumstances, which corresponds to observations on root colonization. Nodule colonization was also higher with AMF isolate BEG 21 than with isolate DD-5, while BEG 21 and DD-5 both belong to the same AMF type (Glo8 which is genetically comparable with *Glomus intraradices*). Glo8 was previously observed to be very abundant in nodules in the field (Scheublin *et al.*, 2004). However, under these experimental conditions no difference in nodule colonization was found between the Glo8 isolates and isolate DD-1, which was a rare AMF type in nodules in the field. Possibly, differences in colonization ability become apparent only when different AMF are in competition with each other.

In conclusion, AMF are able to colonize leguminous root nodules, but AMF-colonized nodules do not fix N. Therefore AMF are not directly interacting with N-fixing rhizobia in the root nodules, and it is likely that AMF colonize senescent nodules. Synergistic interactions in the tripartite legume–AMF–rhizobia symbiosis are probably indirect and mediated via the legume.

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